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THE EFFECTS OF ACUTE AND CHRONIC MORPHINE TREATMENT ON THE PROCESS OF FACIAL NERVE REGENERATION

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SUMMARY

Prominent cellular responses to axonal interruption include enhanced synthesis of RNA and protein in the neuronal perikaryon, and proliferation of reactive Schwann cells. Since morphine has been shown to significantly depress cellular metabolism, we examined its effect on these and other reparative responses underlying nerve fiber regeneration.

Rat facial nerve trunks from saline, acute morphine, and continuous morphine-treated animals were examined by light and electron microscopy at 3, 7 and 14 days after crush injury. The number of axonal sprouts/unit area and the diameters of regenerating axons were quantified at each survival interval.

Both saline-treated and acute morphine-treated facial nerves demonstrated myelin degradation and Schwann cell hypertrophy (at 3 days post-axotomy), sprout outgrowth (at 7 days) and axon maturation and myelination (at 14 days). In the chronic morphine-treated animals, a retardation of the regenerative process was evident. Axon sprout outgrowth and axonal diameters were reduced at 3 and 7 days post-axotomy. In treated 14-day animals, axon diameters were normal; however, significantly fewer axon profiles/unit area were observed. After chronic morphine exposure, Schwann cell hypertrophy and proliferation, as well as myelin debris removal, were inhibited at all survival periods.

INTRODUCTION

In addition to their well-characterized physiological effects, opiates induce marked alterations of CNS biochemistry, including inhibition of RNA[2] and protein synthesis[3,4]. Since other pharmacological agents interfering with various aspects of cellular metabolism influenced the neuronal regenerative response[7,8,14,20], it seemed likely that similar alterations might occur after opiate treatment. We have recently

observed that axotomized facial nucleus neurons were more vulnerable than normal cells to the protein synthesis depression induced by morphine[15]. Furthermore, chronic morphine exposure altered the ultrastructural appearance of the axotomized cells and increased the number of degenerating neuronal profiles[16].

Since enhanced perikaryal synthesis of structural constituents is an essential prerequisite for axon reconstitution[6], drug-induced inhibition of neuronal metabolism might influence the onset and morphological expression of nerve fiber outgrowth. In addition, reductions of RNA and protein synthesis may have a detrimental effect on the proliferation of Schwann cells and other phagocytic elements that normally accompanies anterograde degeneration. If the reactivity and division of such cells are inhibited, myelin degradation and axonal sprout outgrowth are known to be delayed[7]. Thus, in the following investigation, the consequence of morphine treatment on the process of nerve fiber regeneration was examined at various intervals after facial nerve interruption.

MATERIALS AND METHODS

(A) *Animals and preparation*

Forty-two adult male Wistar rats, weighing between 275 and 325 g, were given water and chow (crude protein, 23%) ad libitum. Two days prior to nerve crush, a polyethylene cannula (P.E. 10 tubing, Clay Adams, N.Y.) was inserted into the left external jugular vein[1] for subsequent injections. In 36 animals, the left facial nerve was crushed with a fine forceps 2 mm distal to the stylomastoid foramen. Four unoperated animals and 2 others in which the nerve trunk was exposed served as normal and sham controls. Animals were weighed daily and monitored for return of nerve function (return of eye blink reflex and movement of vibrissae).

(B) *Experiment 1. Acute morphine effects*

Eighteen rats were divided into three survival groups (3, 7 and 14 days post-nerve crush injury). Two hours after nerve crush, 3 rats in each group received morphine sulfate (40 mg/kg i.v.) and 3 controls were administered an equal volume of saline. This morphine dose induced hot-plate analgesia for a period of 1.5–2 h and, in a previous experiment, significantly inhibited protein synthesis in regenerating neurons[15].

(C) *Experiment 2. Chronic morphine effects*

Eighteen rats were divided into three groups (3, 7 and 14 days). Three animals of the 14-day group were administered morphine sulfate throughout the entire survival period, and 3 controls received saline. Rats are extremely refractive to morphine's pharmacological effects[9] and, due to rapid tolerance development, increasingly higher levels of morphine were required to maintain uniform levels of hot-plate analgesia. The initial morphine dose, administered 2 h after nerve crush, was 20 mg/kg i.v.; subsequent doses, however, were increased to 30, 40, 50, 70, 90, 110 mg/kg twice daily injection. A maintenance dose of 110 mg/kg was administered twice daily during the final 7 days. Six animals of the 7-day group were administered saline or morphine at dose levels given to the 14-day group during the first 7 days of treatment. Finally, 6 animals of the 3-day survival group received saline or morphine in doses of 20, 30 and 40 mg/kg, twice daily.

(D) *Tissue preparation*

Facial nerve trunks were fixed in situ using methodology similar to that described by Morris et al[10] and Friede and Martinez[5]. Briefly, animals were anesthetized, incisions carefully reopened, and the exposed nerve completely covered with a mixture of chilled 3% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer. Fixative was continually replaced with fresh stock over a period of 15 min. A 5 mm nerve segment cut proximal and distal to the crush was excised, immersed for 6 h in glutaraldehyde at 4 °C and stored at the same temperature in buffered 5% sucrose, pH 7.4. Tissues were post-fixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated in ethanol followed by propylene oxide and embedded in Spurr.

Five millimeter by 0.5 μm thick longitudinal sections were cut with a Sorval JB-4 microtome and stained with 0.01% toluidine blue. After determining areas of crush, outgrowth and Wallerian degeneration, the longitudinal blockface was cut 1 mm distal to the crush site. This area was removed, rotated 90° and reattached with cyanoacrylate glue to a new block. In this manner, 0.5 μm thick transverse sections in the region of distal axonal regrowth were taken. Stained transverse sections were examined with a Zeiss Ultraphot-3 microscope using oil immersion optics. At each survival interval, the total number of new axons per 10,000 cu. μm of regenerating nerve cross-sectional area was determined. In addition, an adjustable eye-piece micrometer (Bausch and Lomb) was utilized to measure regenerating axonal diameters. Differences between treated and control tissues were tested for significance using a 3 × 3 two-way analysis of variance[22].

Ultrathin sections were also cut from the transverse blockface. These sections were mounted on nickel grids and double stained with aqueous uranyl acetate and lead citrate. Sections were examined with a Philips 300 electron microscope.

RESULTS

Axonal regrowth and remyelination in saline-treated rats

Light microscopic examination

Three days after nerve crush, dramatic morphological alterations, including fragmentation and disintegration of axons and myelin sheaths, were observed in nerve trunks distal to the site of injury. The ordered, circular cross-sectional appearance of fibers in unoperated nerves was replaced by disorganized odd-shaped myelin rings which were embedded in and separated by a matrix of cellular processes. Mitotic figures, often found in the cell matrix (Fig. 1), suggested that cellular poliferation coincided with nerve fiber degradation. At 7 days, significant regenerative changes were observed in regions 1 mm distal to the site of crush. At this interval, the degenerat-

Fig. 1. Mitotic figures (arrows) and degenerating nerve fibers in rat facial nerve 3 days after nerve crush. Taken from a region 1–2 mm distal to the site of injury. 1000 ×.

ing myelin rings (observed at 3 days) had broken up into darkly stained granules which filled reacting Schwann cells. Numerous pale-staining axon sprouts had traversed the site of injury and were organized into discrete groups or clusters (Fig. 2a). (Axon sprout diameters and density are presented in Table I.) At 14 days, the regenerating axons were larger and well myelinated (Fig. 3a, Table I). Peri- and endoneurial connective tissue had reformed; however, there was a noticeable absence of myelin debris and little evidence of mitotic activity.

Electron microscopic examination

At 3 days, hypertrophic Schwann cells formed cellular 'collars' around fragmentated axon and myelin membranes. Axonal sprouts containing neurofilaments, mitochondria and a pale-staining axoplasm were found crowded between the surviving basal lamina and the plasma membrane of debris-laden Schwann cells. Macrophages, distinguished by the absence of basement membrane and the presence of lysosomal bodies, were occasionally observed in the endoneurial matrix. Seven days after nerve crush, Schwann cells containing osmiophilic debris and lipid-filled vacuoles were associated with well-developed axon sprouts. Many of the new axons had 2–5 layer thick myelin sheaths (Fig. 4a). Elongated fibroblasts interspersed between axon–Schwann cell groups compartmentalized the regenerative outgrowth. At 14 days, the the myelin sheath thickness of the new axons approached one-half of that observed in unoperated nerves. The Schwann cells remained enlarged and most displayed hyper-

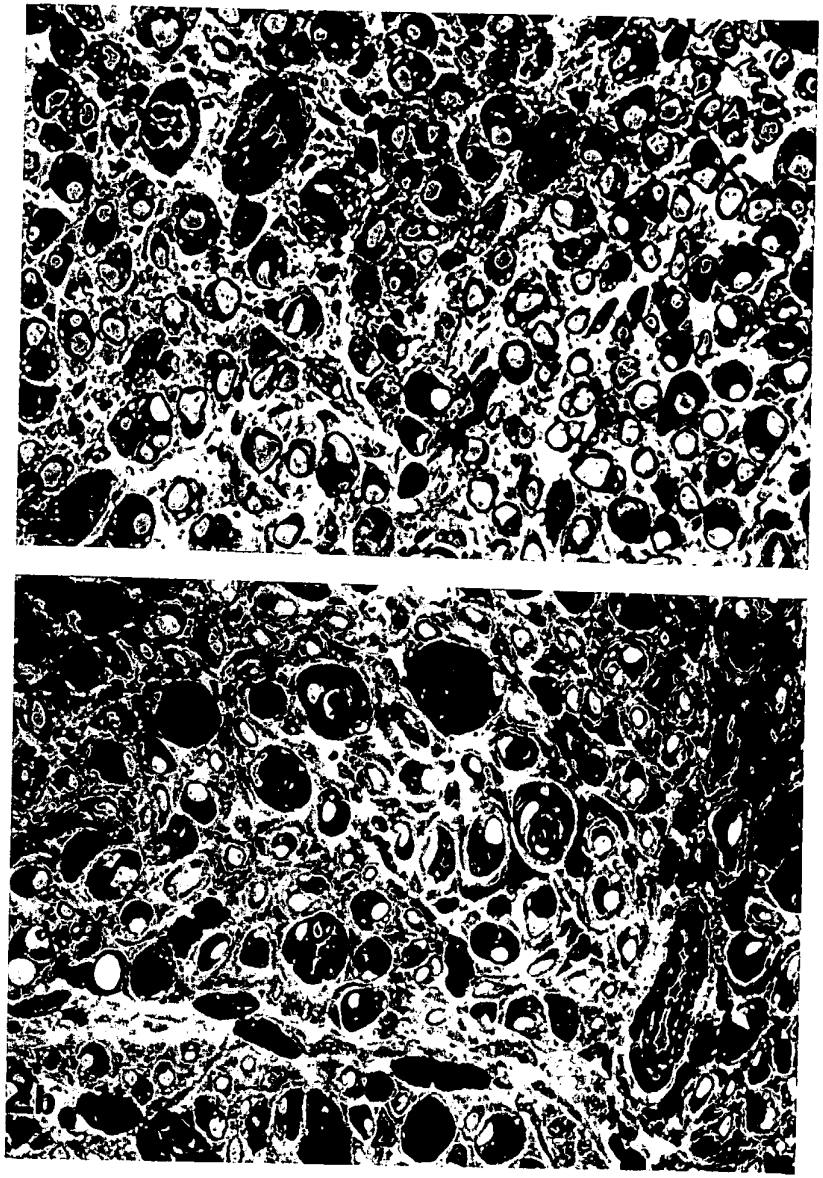

Fig. 2. Rat facial nerve 7 days after nerve crush; taken from a region 1 mm distal to the site of injury. 500 ×. Transverse sections from (a) saline-treated and (b) chronic morphine-treated animals.

TABLE I

Comparison of axonal sprout outgrowth and diameters of new axons at various intervals after facial nerve crush injury in saline-treated and acute and chronic morphine-treated rats

	Saline-treated		*Acute morphine-treated*		*Chronic morphine-treated*	
	*Axon profiles**	*Diameter***	*Axon profiles**	*Diameter***	*Axon profiles**	*Diameter***
3 days post-nerve crush	11 ± 2.4	5.6 ± 1.4	12.4 ± 3.1	5.9 ± 2.3	2.0 ± 1.5§	5.0 ± 1.0
7 days post-nerve crush	199 ± 11.6	8.9 ± 1.9	206 ± 9.8	8.7 ± 1.7	141 ± 14.6§	6.1 ± 1.3§§
14 days post-nerve crush	240 ± 10.3	10.9 ± 2.1	237 ± 13.3	10.1 ± 1.95	179 ± 12.6§	8.8 ± 1.6
Unoperated facial nerve	272 ± 12.4	13.4 ± 3.0				

* Values represent the mean of the means within treatment groups and the standard error of the mean (n = 3; 10 observations/n). Observation = number of axonal profiles/10,000 square microns.

** Axon profile diameters in microns: values represent the mean of means and standard error of the mean (n = 3; 100 measurements/n).

§ Significantly less than saline-treated controls at the same survival interval period ($P < 0.01$); §§ ($P < 0.05$).

trophied profiles of Golgi and endoplasmic reticulum (Fig. 4c). Partial return of facial nerve function coincided with nerve fiber maturation. Although vibrissal movement was not evident, return of the eye blink reflex was observed in 5 of the 6 saline-treated animals.

Axonal regrowth and remyelination after acute morphine treatment

An acute administration of morphine sulfate did not influence the process of axonal regrowth and remyelination. Table I illustrates that in treated animals, axonal diameters and density at both 7 and 14 days following nerve crush were not statistically different from values observed in saline controls. Drug-induced alterations of axon sprout and Schwann cell morphology were not evident. Partial return of nerve function (eye blink reflex) was observed in 2 of 3 animals.

Axonal regrowth and remyelination after chronic morphine treatment

Light microscopic examination

In treated animals, a retardation in the development and extent of Wallerian degeneration was observed. Axon and myelin sheath breakdown was less pronounced and many of the injured fibers retained a normal (unoperated) morphology. In transverse sections, decreased cellularity and mitotic figures, as well as few axonal sprouts, were observed. At 7 days, the number of sprouts per unit area and diameters of individual axons were significantly less than values observed in saline- or acute morphine-treated animals (Table I). Moreover, those sprouts present were interspersed between

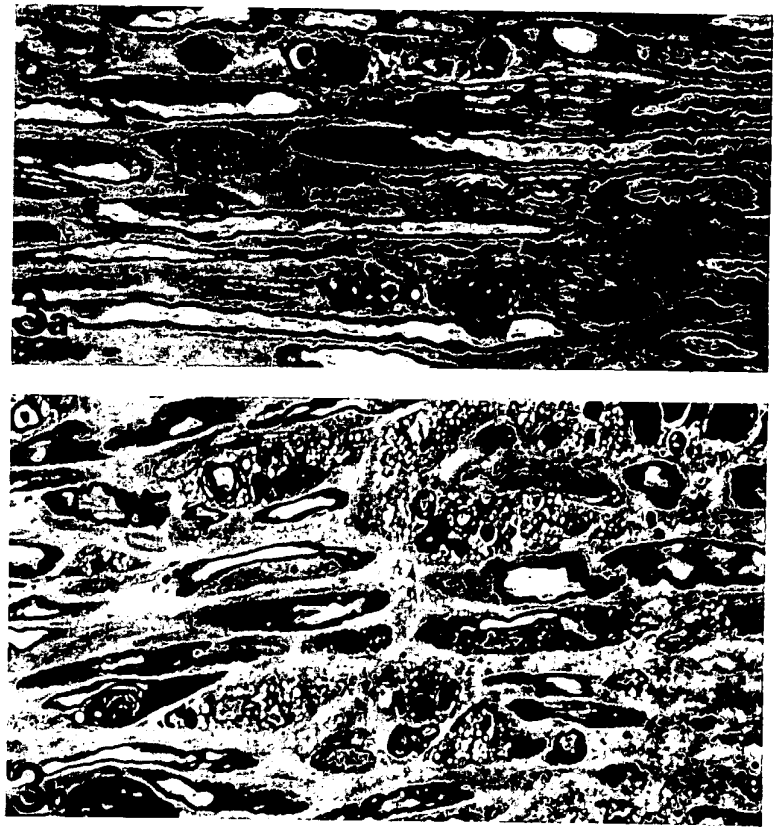

Fig. 3. Rat facial nerve 14 days after nerve crush; taken from a region 1–2 mm distal to the site of injury. 500 ×. Longitudinal sections from (a) saline-treated and (b) chronic morphine-treated animals.

large amounts of myelin debris, and in transverse sections appeared disorganized and rarely packed into discrete groups (Fig. 2b). After 14 days of continuous morphine exposure, the regenerated axons remained disoriented and separated by large deposits of partially digested myelin debris (Fig. 3b). In transverse sections, reduced numbers of regenerated axons were separated by debris-laden cells and unstained loose connective tissue. However, measurements of axonal diameters revealed no size differences between treated and control groups (Table I).

Electron microscopic examination

Nerve fiber degeneration was evident at the early survival interval; hypertrophy of Schwann cells, and phagocytosis and digestion of degenerating debris, however,

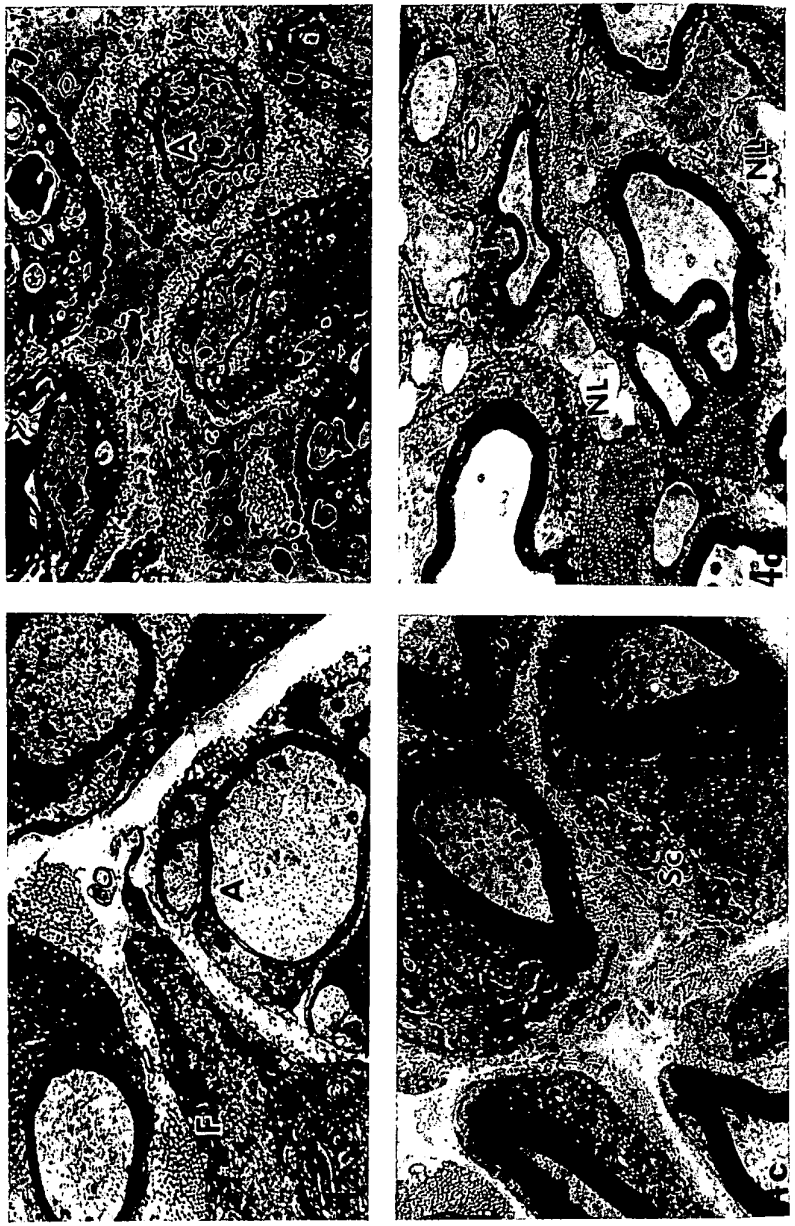

Fig. 4. Regenerating nerve fibers in rat facial nerve at various time intervals following nerve crush injury. Sections from (a) saline-treated and (b) chronic morphine-treated animals 7 days after crush; and from (c) saline-treated and (d) chronic morphine-treated animals 14 days after crush. 5900 ×. A, axons; F, fibroblast; Sc, Schwann cell; NL, neutral lipid.

were less pronounced than that occurring in saline-treated controls. At 7 days, axon sprouts separated by debris-filled cells were surrounded by thin and irregular myelin sheaths (Fig. 4b). Significant compartmental organization of the new axons by endoneurial fibroblasts was less obvious.

Although axons present at 14 days contained normal axoplasm, myelin sheaths were reduced in thickness. Schwann cells and fibroblast processes were often filled with undigested myelin debris or lipid droplets (Fig. 4d), material rarely observed in saline controls. Axons and debris-laden cells were embedded in a dense collagen matrix. Return of facial nerve function was not evident.

DISCUSSION

Crush lesions of the facial nerve interrupted axonal continuity and elicited stereotyped (Wallerian) degeneration. As in previous investigations[10,11,19], Schwann cells reacting to the progressive deterioration of distal segment axons rapidly altered their structure and function and reverted into actively proliferating phagocytes. Such alterations subserved the interrelated processes of debris removal and guidance of axon outgrowth. These new functions were evident at early survival intervals, when hypertrophic Schwann cells actively digesting remnants of degenerating nerve fibers were observed simultaneously accepting new axonal sprouts from the proximal segment. At later survival intervals, the newly established axons increased their cross-sectional diameter, became myelinated and demonstrated return of function.

Chronic morphine exposure significantly inhibited the onset and magnitude of axonal regeneration. Strikingly similar neuropathological alterations have been reported in neonatal rat pups after exposure to methadone[17]. When sural nerves of treated pups were examined by electron microscopy, diminished numbers of myelinated fibers, reductions of axonal diameter, and a pronounced increase in endoneurial collagen were observed (Smith, personal communication, and ref. 17). Although the outgrowth of new axons is dependent upon materials transported from sites of synthesis in the neural soma, there is little evidence to suggest that opiates interfere with the process of intra-axonal movement. Morphine administration did not alter rates of fast or slow transport in optic nerve axons[21]. After single[4,12] or multiple[4,21] doses of morphine, however, significant depressions of neuronal protein synthesis were reported. Furthermore, when compared with uninjured cells, the magnitude of morphine-induced protein synthesis depression was greater in axotomized neurons[15]. Thus, in the present investigation, delayed axon outgrowth and reduced sprout caliber may reflect similar decreases of structural protein synthesis in injured cell bodies. In addition, reductions in axonal density seen at later survival intervals may correlate with the increased cell death observed when axotomized neurons were continuously exposed to morphine[16]. Although some stereospecific opiate binding has been reported in facial nucleus[13], it remains unclear whether these receptors or morphine's ipecac-like interaction with protein translation[2] mediate these metabolic and growth retarding effects.

Peripheral drug effects expressed at the site of crush injury may also antagonize

the regenerative response. During early regenerative intervals, reductions in the number of mitotic figures and actively phagocytic cells suggested that morphine inhibited Schwann cell division and interfered with the process of nerve fiber degradation. This effect resembled the inhibitory activity of mitomycin C[7,8] and colchicine[14], in which retardation of the morphological changes associated with Wallerian degeneration and axonal regrowth was related to reduced Schwann cell proliferation. Evidence of peripheral opiate effects were reported in regenerating salamander limb buds[18], in which profound inhibitions of uridine and thymidine incorporation and reduced nerve growth were observed after chronic methadone exposure. Drug-induced inhibition of phagocyte proliferation may explain why axons sprouted into areas of minimal myelin degradation during early survival intervals and also why debris-laden cells were present at later intervals in chronic morphine-treated animals.

Single injections of morphine at dose levels which significantly reduced protein synthesis in regenerating neurons did not inhibit nerve fiber degradation and axonal regrowth. It seems likely that significant alterations of the regenerative response and delayed return of function are dependent upon continuous drug-induced biosynthetic depression and possible cumulative toxicity. Moreover, observations of axonal loss and persisting defects in debris removal indicate that chronic morphine exposure may induce permanent neurological deficits.

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